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13. ABSTRACT *(Maximum 200 Words)*

The hyaluronan binding protein RHAMM is located on the cell surface in the cytoplasm and the nucleus as well as lamellae and in these characteristics resembles the morphogenic branching factor epimorphin. Our goal was the identification and characterization of the interplay between RHAMM and epimorphin during mammary gland morphogenesis and, for the future, breast tumor progression. In order to begin to identify and investigate a function for RHAMM during epimorphin-regulated mammary gland morphogenesis we analyzed RHAMM expression in virgin and pregnant mice and studied the effect of RHAMM deficiency on *in vivo* and *in vitro* branching morphogenesis. RHAMM is expressed by breast epithelial cells, as detected by RT-PCR and western blot analysis and expression increases during pregnancy as shown by RT-PCR analysis, immuno staining and in situ hybridization. Mammary glands isolated from RHAMM -/- mice show reduced secondary branching compared to mammary glands isolated from wild type (wt) mice. Absence of RHAMM due to genetic deletion or inhibition of RHAMM function by function blocking reagents e.g. antibodies or recombinant protein fragments, leads to enhanced branching in *in vitro* assays. This difference between *in vivo* and *in vitro* results suggests that the *in vitro* environment might provide factors e.g. growth factors, which are limiting in the *in vivo* environment, possibly due to the loss of the RHAMM gene. Resurfacing of scratch wounds of breast epithelial cell monolayer is enhanced in the presence of function blocking anti-RHAMM antibodies and regulation of cell motility might be one mechanism by which RHAMM influences branching morphogenesis.

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INTRODUCTION

Hyaluronan is a negatively charged polysaccharide ubiquitously associated with the extracellular matrix that is synthesized in mammals by three hyaluronan synthases (HAS1,2,3) (1). These enzymes are unusual glycosyltransferases as they occur at the plasma membrane rather than within the golgi (2). They encode multiple membrane spanning sequences and pass through the plasma membrane several times. The UDP-sugar binding regions occur within the cytoplasmic portion of HAS1,2,3 and thus hyaluronan is polymerized in the cell cytoplasm. It is exported from the cell but the mechanism for this is still controversial. By analogy to lower eukaryotes, hyaluronan has been proposed to be released through pores formed by HAS and cardiolipin aggregates (3). Whether or not the three HAS enzymes have overlapping functions has not yet been resolved. However, genetic deletion of HAS 2 alone results in embryonic lethality (4). One interpretation for this result is that the HAS enzymes are differentially regulated. For instance, PDGF stimulates expression of HAS 2 but not HAS 1 or 3 (5).

Hyaluronan has been linked to regulation of motility and proliferation during wound repair and neoplastic conversion (1). In addition, hyaluronan has also been implicated in the branching morphogenesis of the salivary, prostate and uterine glands (6,7) and may regulate involution following weaning (8). Evidence supporting these roles include: a) enhanced production of hyaluronan in wounds, around tumors (9) and within clefts of branching tissues *in vivo*; b) the ability of highly purified hyaluronan to stimulate the motility/proliferation *in vitro* and to activate signaling cascades particularly through ras and erk (10), which also is required for branching morphogenesis; and c) reduced motility/morphological transformation of cardiac endothelial cells that do not express hyaluronan synthase 2 due to genetic deletion, and rescue of these functions by the addition of purified hyaluronan (4). The ability of hyaluronan to promote these cell functions depends in part upon its molecular weight. In general, lower molecular weight fragments of hyaluronan are more active than high molecular weight hyaluronan in promoting cell signaling (10). However, there are exceptions and oligosaccharides approximating the size required for binding to a single site on cellular hyaladherins can apparently compete with larger fragments of hyaluronan for receptor binding and thus inhibit signaling responses (9). Nevertheless, a molecular rationale for the effectiveness of fragments vs. higher molecular weight hyaluronan has not been identified. Furthermore, the functions of the hyaluronidases that have been cloned to date have not been well characterized (1).

Hyaluronan or its fragments appear to exert most of its effects on the above cell functions through its association with both extracellular and cellular hyaluronan binding proteins termed hyaladherins (10). Both groups of hyaladherins are required for normal tissue function. Thus, genetic deletion of link protein results in neonatal death while genetic deletion of the cellular hyaladherin CD44 results in aberrant inflammatory responses and, depending upon the mouse background, premature involution(1).

Cellular hyaladherins can be grouped into at least two classes of proteins, trans-membrane receptors and "itinerant" proteins that occur in multiple subcellular compartments including the cell surface (10). CD44 and LYVE 1 are examples of the

first category. RHAMM, which was the first cellular hyaladherin to be isolated, is an example of the second category.

RHAMM occurs as multiple protein forms that result from alternative splicing of mRNA as well as as yet uncharacterized post-translational mechanisms. Isoforms have different functions and can occur in different subcellular compartments. For instance, full length RHAMM decorates the microtubules and actin filaments while a transforming isoform which lacks N-terminal sequence and is most abundantly expressed in trafficking white cells, aggressive cancers and subconfluent cultures responding to injury primarily associates with cell ruffles and lamellae (10). The presence of RHAMM at the cell surface has been controversial since the RHAMM gene does not encode either a signal peptide to direct export through the golgi-ER or a membrane spanning sequence to permit generation of signals. Nevertheless, RHAMM is expressed at the surface of non-adherent cells and sub-confluent adherent cells, as detected by FACS, scanning electron microscopy and antibody/peptide inhibition studies (10). Cell surface RHAMM has recently been designated as CD168 by the leukocyte-typing workshop. RHAMM thus resembles an intriguing group of proteins that lack signal peptides but are exported in response to specific stimuli. These include bFGF, hox transcription factors, HIV tat, epimorphin annexins and heat shock proteins. The mechanisms of export have been best documented for bFGF. BFGF is exported from the cell in response to heat shock (11) and this process requires the interaction of bFGF with specific protein binding partners (11). Through these interactions bFGF is proposed to associate with annexins at the inner plasma membrane. Annexins and their binding partners are flipped to the exterior of the cell through as yet undescribed mechanisms, in response to injury. A similar "flip-flop" phenomenon has been described in erythrocytes where lipid probes such as lysophosatidylcholine and palmitoylcarnitine at the inner plasma membrane "flip" to the outer leaflet in response to calcium ion flux generated by ionophores (12). An alternative possibility is that RHAMM and other itinerant cellular hyaladherins may be exported by "piggy-backing" on the newly synthesized hyaluronan chains that are extruded from the cell, possibly, as noted above, via pores in the plasma membrane. Finally, it is intriguing that many of the signal peptide-less proteins, including RHAMM, are homologous to proteins associated with vesicle transport.

Cell surface RHAMM, like CD44, binds to both hyaluronan and to fibronectin, and these interactions result in activation of signaling cascades particularly through src and erk kinase (10). The association of RHAMM with hyaluronan and fibronectin, however differs from that of CD44. Thus, RHAMM binds to hyaluronan via basic amino acids motifs while CD44 associates with this polysaccharide via a larger protein module termed the link module (13). RHAMM binds to the CS-1 region of fibronectin (unpublished data) while CD44 binds to the heparin binding region II of fibronectin (14). Interestingly, both CD44 and RHAMM regulate similar signaling cascades and co-associations between the two hyaladherins have been observed in aggressive breast cancer cell lines that express mutant active ras. Antibody blocking studies indicate that RHAMM is required for hyaluronan-mediated activation of src kinase, protein tyrosine phosphorylation/dephosphorylation of FAK and activation of the erk kinases (10). Furthermore, blocking cell surface RHAMM function with antibodies or peptide

mimetics inhibits cell motility (15), progression through G2M of the cell cycle (16) and tubule formation by endothelial cells (17). We have proposed that cell surface RHAMM is a peripheral protein that associates with integral membrane receptors such as CD44 and PDGFR (18). For instance, cell surface RHAMM is required for maximal protein tyrosine phosphorylation and activation of erk kinase by PDGF (18). Furthermore, RHAMM-/- cells are unable to sustain activation of erk kinase in response to PDGF *in vitro* (unpublished data). However, the specific function of RHAMM-hyaluronan or RHAMM-fibronectin interactions in signaling through integral receptors is not at all clear. Furthermore, which RHAMM isoforms are exported to the cell exterior has not yet been defined.

Intracellular RHAMM has been proposed to act as a microtubule-stabilizing protein (MAP) (19) but there is little evidence to support this proposal. For instance, RHAMM encodes multiple SH2 and SH3 binding sequences, phospho-acceptor sites, and other protein recognition sequences that predict it may function as a scaffold protein linking signaling complexes to the cytoskeleton much like paxillin and vinculin (10). In support of this proposal, RHAMM contains a near perfect consensus erk docking sequence and has been shown to associate with erk kinase in fibroblasts and recombinant RHAMM binds directly to erk 1 kinase (unpublished data). Intracellular RHAMM isoforms exert differential effects on the cytoskeleton. The N-terminal truncated form of RHAMM that is transforming in fibroblasts, enhances microtubule turnover, focal contact turnover and remodelling of the actin cytoskeleton (20). The effect of this RHAMM form requires erk kinase activity. In contrast, full-length intracellular RHAMM form does not exert these effects and its function within the cytoskeleton is not yet clear.

RHAMM and CD44 hyper-expression have been linked to disease states in the human. In particular, both RHAMM and CD44 are hyper-expressed in breast cancer and closely linked to progression and prognosis of this disease (9). In these studies, RHAMM protein was detected in both the carcinoma cells and stroma surrounding the tumor. Both RHAMM and CD44 are over-expressed in aggressive breast cancer cell lines (e.g. MDA-MB-231) compared to less aggressive tumor cell lines (e.g. MCF-7). The expression of both hyaladherins are regulated through activated ras (21,22) and this appears to involve primarily post-translational methods for protein stabilization.

CD44 variant forms (v3 and v6) are expressed in myo-epithelial and acinar cells of salivary and mammary glands (6, 19, 23) and genetic deletion of CD44 on a CD-1 background results in accelerated postpartum involution and impaired maintenance of lactation. Further, animals exhibit reduced fertility. Interestingly genetic deletion of CD44 on a C57BL/6 background does not exhibit these defects. The expression of RHAMM in normal breast tissue and whether or not it plays a role in breast tissue function has not previously been described and is the subject of this study.

BODY

In order to assess the role for RHAMM in breast tissue morphogenesis and function, two approaches were undertaken. In the first C57BL/6 RHAMM -/- mice were prepared to permit assessment of the consequences of loss of RHAMM to branching morphogenesis *in vivo* and *in vitro*. For the second approach, immortalized breast epithelial cell lines were used to assess the effects of RHAMM antibodies on cell proliferation, branching and motility *in vitro* and to dissect the role of RHAMM in regulating signaling pathways that control these cell functions.

A. RHAMM -/- mice

The strategy for deleting RHAMM in C57BL/6 mice is shown in Figure 1. C57BL/6 mice were chosen for genetic manipulation since the most thoroughly characterized CD44-/- genetic deletion is also on a C57BL/6 background (24). It is likely that any phenotype associated with RHAMM deficiency may be amplified using CD44-/- X RHAMM-/- mice. For instance, RHAMM expression is up-regulated in these CD44-/- animals (D. Naor, personal communication). Exon 8 through 16 of the RHAMM gene were removed and a hprt marker flanked by loxP sites was inserted. This strategy would theoretically result in the expression of a N-terminal truncated RHAMM comprised of exon 1-7. Exons 17-18 would not be translated into RHAMM protein as they are out of frame. A minor mRNA (barely detectable) transcript corresponding to this size was amplified in RHAMM -/- but not wild-type tissue (data not shown). We therefore prepared an antibody to exon 7 to assess the protein expression from this mRNA transcript.

The expression of RHAMM mRNA and protein in wild-type and RHAMM -/- tissue are shown in Figure 2 (A-C). RHAMM mRNA and protein expression of several cultured breast mammary epithelial cell lines are shown in Figure 2(D-E). A mutant active H-ras transformed cell line (C3) was used as a positive control since these cells have previously been shown to express large amounts of RHAMM (24). RHAMM -/- tissue does not express the full length RHAMM mRNA transcript (Fig. 2 A) or protein (Fig. 2 B) as expected while both mRNA and protein are detectable in the wild-type tissues. Immortalized RHAMM -/- embryonic fibroblasts infected with an empty vector do not express detectable RHAMM using an immunofluorescence assay while cells infected with full length RHAMM do express detectable protein (Fig. 2 C). These results show that the reagents used to detect RHAMM are specific. All three of the mammary cell lines express RHAMM mRNA (Fig. 2 D) and protein (Fig. 2 E) although EPH4 and SCg6 appear to express slightly more mRNA and protein than SCp2 cells. These results show that mammary epithelial cells can express RHAMM mRNA and protein. All cell lines are expressing full length (95 kDa) protein under the culture conditions used.

To determine whether or not RHAMM is expressed during mammary gland morphogenesis, mRNA were isolated from mammary glands of pubescent, pregnant and post-parturition CD-1 mice and analyzed using RT-PCR. As shown in (Fig. 3 A), older, but still pubescent, virgin, mice express higher levels of RHAMM than younger virgins.

This variation of expression between virgin mice could be the result of different hormone levels. If RHAMM expression is influenced by blood hormone levels and changes during estrous cycle has to be determined. During pregnancy of 8 weeks old mice, RHAMM expression is sustained and, if the E-cadherin loading control is accurate, increases during the last days of gestation. When lactation begins RHAMM expression drops. E-cadherin was used as a marker for epithelial cells and hprt as a "housekeeping" loading control. These results show that RHAMM expression is regulated in mammary tissue during late pubescence and pregnancy but RHAMM is not expressed during lactation.

The expression of RHAMM mRNA and protein was then assessed in virgin, and pregnant (15 days) C57BL/6 mice, the background used for the RHAMM knock out. Vimentin, E-cadherin and/or beta actin were used as loading controls. Mutant active H-ras transfected cells were used as positive controls. As shown in Figure 4(A), virgin C57BL/6 mice express RHAMM mRNA or protein in mammary tissue at a very low level while pregnant (day 15) mice show increased RHAMM mRNA expression (Fig. 4 A) and full length protein (95 kDa, Fig. 4 B) can be detected. These results show that pregnant C57BL/6 mice express RHAMM in mammary tissue, consistent with the result obtained in CD-1 mice. In order to identify the cell type(s) in mammary gland tissue that express RHAMM mRNA transcripts, in situ hybridization was conducted using frozen sections of ovary tissue (Fig. 5 A) as a positive control, and in mammary tissue taken from day 1 of parturition when RHAMM expression is still high (Fig. 5 B). RHAMM mRNA transcripts were identified in the granulosa cells of the ovary. Epithelial cells, but not stromal cells express RHAMM mRNA at this particular stage in mammary gland morphogenesis.

To begin identify a possible function of RHAMM during mammary gland morphogenesis, whole mounts of mammary glands of 7 wk C57BL/6 virgin wildtype (wt) and RHAMM -/- (ko) mice were prepared. The mammary glands of virgin RHAMM -/- mice exhibit less secondary branching than wild-type glands (Fig. 6). However, as determined by vaginal smears the wt mouse was in metestrous and the ko mouse in proestrous, complicating interpretations. Nevertheless, these very preliminary results suggest a secondary branching defect in RHAMM -/- mice but additional mice have to be analyzed and the blood hormone levels determined. The upregulation of RHAMM expression during pregnancy suggests a function for RHAMM during alveoli development and this possibility is also being investigated. Breeding of homozygous females with homozygous males would be optimal for obtaining homozygous offspring but was rarely successful and the RHAMM -/- line had to be maintained by breeding of heterozygous mice. This suggests a fertility problem in either male or female homozygous mice. To investigate this in more detail homozygous males were allowed to mate with wt females and homozygous females were allowed to breed with wt males. Whereas both combinations produced a comparable number of litter, the litter sizes were significantly smaller ($P<0.01$) when homozygous males were crossed with wt females. Because both homozygous males and females produce offspring when crossed with wt mice, the inefficient breeding between homozygous animals might be the result of a small fertility reduction in both homozygous males and females that allows breeding with wt but not with homozygous animals.

B. In vitro Experiments

Organoids prepared by partial trypsin and collagenase digestion of mammary gland tissue recapitulate parts of the *in vivo* branching morphogenesis *in vitro* in the presence of collagen and growth factors. This *in vitro* system can be easily manipulated by e.g. use of different matrices and growth factors and therefore allows the identification of signaling pathways influenced by RHAMM. Figure 7 shows the *in vitro* branching of mammary organoids taken from wild-type (wt) and RHAMM -/- (ko) mice cultured in the presence of EGF and collagen I (cellagen). Surprisingly, branching of RHAMM -/- organoids *in vitro* is increased compared to wild-type organoids. Two additional experiments showed the same tendency but the difference between wild-type and -/- organoids was not as obvious as in the first experiment. This variation between experiments is likely due to differences in the mammary gland morphology and hormone status between individual mice. Therefore a larger number of mice will be analyzed prior to reconcile the *in vivo* (Fig. 6) vs. these *in vitro* results.

In order to obtain a model system that circumvents heterogeneity intrinsic to animal models and allows easier manipulation of tissue, we prepared aggregates of breast epithelial cell lines and analyzed branching under the same culture conditions as used in organoid branching experiments. We manipulated cell surface RHAMM function by blocking antibodies. These antibodies were shown by western and immunofluorescence assays to be specific for RHAMM (Fig. 2). As shown in figure 8, EPH4 cells (which express RHAMM, see Fig. 2) exposed to EGF branch as reported (25). The addition of either function blocking antibodies (e.g. inhibits motility of fibroblasts) or a recombinant RHAMM fragment, previously shown to block mutant active ras-mediated transformation in fibroblasts, enhance branching in these cells. These results are thus similar to those observed with RHAMM -/- mammary organoids.

The effect of the same RHAMM function blocking reagents on breast epithelial scratch wound response in the presence of EGF was examined next. As shown in figure 9, the addition of function blocking RHAMM reagents accelerated closure of scratch wounds. These results suggest that blocking RHAMM function in breast epithelial cells enhances cell functions such as motility. This is in contrast to fibroblasts where the same RHAMM function blocking reagents inhibit closure of scratch wounds.

As noted above, cell surface RHAMM is not an integral membrane protein and therefore most likely associates with integral membrane receptors to affect their ability to signal. We have previously shown that RHAMM co-associates with PDGFR and with CD44. Here, it can be seen that RHAMM also co-associates with N-cadherin in fibroblasts and that RHAMM/N-cadherin complexes also contain Beta catenin and erk 1,2 kinase. We have unsuccessfully tried to co-immunoprecipitate RHAMM with E-cadherin but there may be technical reasons for this failure. A recent report noted activation of src kinase through EGFR causes shedding of E-cadherin, which would then permit cell scattering. We have previously shown that RHAMM transiently activates src kinase and that src-/-

fibroblasts do not respond to either RHAMM agonist reagents or to hyaluronan, providing a rationale for these effects of RHAMM in epithelial cells.

KEY RESEARCH ACCOMPLISHMENTS

A. Cementing a research collaboration, which brings new expertise in area of hyaluronan and hyaladherins to the Bissell lab and knowledge of physiological processes in mammary gland morphogenesis to the Turley lab. This will collectively permit novel analysis of as yet unknown physiological functions of hyaluronan and receptors for this polysaccharide.

B. Obtained encouraging preliminary data that suggests that loss of one hyaluronan receptor, RHAMM, results in a branching defect.

REPORTABLE OUTCOMES

Trained key postdoctoral fellow, Dr. C. Toelg, who has considerable molecular and transgenic mouse expertise in key cell biology and other techniques such as fat pad clearing, in situ hybridization, real time PCR, whole mounts of mammary glands and three dimensional cell culture techniques.

CONCLUSIONS

In order to identify possible functions of hyaluronan and the hyaluronan binding protein CD44, during mammary gland morphogenesis we analyzed RHAMM expression in mammary glands of virgin and pregnant mice. Preliminary PCR data suggest up-regulation of RHAMM expression during pregnancy. At the onset of lactation RHAMM is expressed by epithelial cells as shown by in situ hybridization and immunostaining. Because the amount of epithelial cells in the mammary gland increases during pregnancy the observed increase of RHAMM expression in the mammary gland might be due to an increased percentage of epithelial cells or increased expression in epithelial cells. To investigate these possibilities we used E-cadherin as marker for the epithelial cell population. Around parturition RHAMM expression returned to low levels as seen in virgin mice although E-cadherin was still present at high levels, suggesting a regulation of RHAMM expression independent of the amount of epithelial cells.

Whole mount analysis of RHAMM -/- and wt mammary glands of virgin mice suggested reduced secondary branching in RHAMM ko mice. The mice used in this analysis were at different stages of the estrous cycle, therefore the observed difference in branching pattern might be due to a difference in hormone levels. To confirm a role of RHAMM

during branching morphogenesis additional whole mounts will be prepared and blood hormone levels will be analyzed. Because RHAMM expression increases during pregnancy, suggesting a role for RHAMM in alveolar development, mammary glands of pregnant mice will be included in this analysis.

Whereas RHAMM deficiency resulted in reduced *in vivo* branching, the opposite effect was observed *in vitro*. Organoids isolated from RHAMM -/- mice showed enhanced branching in the presence of collagen I and EGF compared to organoids isolated from wt mice. During *in vitro* branching, factors like EGF and insulin are present at high concentrations, whereas the same factors are limited during *in vivo* morphogenesis. This might explain the difference between *in vivo* and *in vitro* results. This possibility could be investigated by the use of different growth factors, growth factor concentrations, and alternative matrices e.g. matrigel instead of collagen.

In vitro branching analysis of clustered mammary epithelial cells in the presence of RHAMM blocking reagents e.g. blocking antibodies or recombinant protein fragments showed the same tendency as the *in vitro* branching of RHAMM -/- organoids. Because the RHAMM blocking reagents only interfere with the function of cell surface RHAMM suggests that this particular subpopulation of RHAMM is involved in mammary gland morphogenesis.

RHAMM blocking reagents accelerated the resurfacing of scratch wounds in an epithelial cell monolayer. This suggests that RHAMM influences epithelial cell behavior like cell motility. If this explains the *in vitro* branching results or if RHAMM also influences cell proliferation or apoptosis has to be investigated.

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FIGURE LEGENDS

Fig. 1:
Strategy used for the deletion of RHAMM in ES cells
Exons 8 to 15 of the murine RHAMM gene were replaced by a hprt selection marker which was flanked on both sides by genomic sequences that allowed integration into the ES cell genome by homologous recombination.
After integration, splicing of exon 7 to exon 17 of the RHAMM transcript results in the expression of a small, truncated transcript.

Fig. 2:
RHAMM is expressed in mammary epithelial cell lines.
A. To confirm specificity of RHAMM PCR total RNA was isolated from Rhamm -/- and wt spleen and subjected to RT PCR analysis using oligo dT and RHAMM sequences as primer. Amplification of β actin transcripts was used as loading control. Ras transformed T101/2 cells (C3 cells) expressing RHAMM were used as positive control. For the negative control the RT reaction was performed without the addition of enzyme.
B. Protein extracts were prepared from RHAMM -/- (ko) and wt spleen and seperated by SDS PAGE. Anti RHAMM anibodies raised against a N-terminal truncated GST fusion protein in rabbits was used in western blot analysis. Incubation of the same blot with anti β actin antibodies was used as loading control. Protein extract from ras transformed cells (C3) were used as positive control.

C. immortalized fibroblasts isolated from RHAMM -/- mice were transfected with either an empty vector as negative control or a construct allowing RHAMM expression. Immunofluorescence staining was performed with anti RHAMM antibodies.
D./E. RHAMM expression of the immortalized mammary epithelial cell lines EPH4, SCg6, SCp2 was analyzed by RT PCR and western blot analysis as described in A. and B.

Fig. 3: RHAMM is expressed in mammary glands of CD-1 mice.
Total RNA was isolated from mammary glands isolated at different time points during puberty, gestation , involution and at parturition (P) and lactation (L). RHAMM, E-cadherin and HPRT expression were analyzed by RT PCR.

Fig. 4: RHAMM is expressed in mammary glands of C57BL/6 mice.
A. Total RNA was isolated from mammary glands from 8 weeks old virgin mice and at day 8 (P8), day 15 (P15) and parturition of gestation. RHAMM, vimentin and E-cadherin expression were analyzed by RT PCR.
B. RHAMM and E-cadherin protein expression in mammary glands from 8 weeks old virgin and 15 days pregnant mice was analyzed by western blot analysis. β actin protein expression was used as loading control.

Fig. 5: RHAMM is expressed by granulosa cells in ovaries and by epithelial cells in the mammary gland.
Frozen sections of ovaries isolated from 8 weeks old virgin mice and mammary glands isolated at parturition were analyzed by in situ hybridization.

Fig. 6: Mammary gland whole mounts of 6 months old wt and RHAMM -/- (ko) mice.
Mammary glands, including the lymph node were stained with carmine.

Fig. 7: RHAMM -/- organoids show increased *in vitro* branching compared to wt organoids.
Mammar glands were isolated from 6 months old virgin mice and partial digested with trypsin and collagenase. The resulting organoids were embedded in collagen I matrix containing EGF and insulin. The pictures were taken 3 days after embedding of the organoids.

Fig. 8: *In vitro* branching of mammary epithelial cell clusters is stimulated by anti RHAMM antibodies and recombinant RHAMM protein fragments.
Clusters of mammary epithelial cells were embedded in collagen I matrix containing EGF and insulin. Branching in the presence of either anti RHAMM antibodies or recombinant

RHAMM protein fragments was enhanced compared to branching in the presence of IgG. Matrix without EGF (-EGF) was used as negative control.

Fig. 9:
RHAMM function blocking reagents stimulate closure of scratch wounds of epithelial cell monolayer.
Monolayer of mammary epithelial cells were scratch wounded with a pipette tip. Recovering of the scratch wound by epithelial cells was analyzed in the presence of either anti RHAMM antibodies, recombinant RHAMM protein fragments or anti E-cadherin antibodies. SCp2 cell migration into the scratch wound is stimulated whereas migration of fibroblastic SCg6 cells is decreased by RHAMM function blocking reagents.

Fig. 10:
RHAMM co-associates with N-cadherin, β-catenin and ERK in fibroblasts.
Either N-cadherin or β-catenin were immunoprecipitated from protein extracts prepared from fibroblasts. The precipitated complexes were seperated by SDS PAGE and probed with either anti RHAMM or anti erk antibodies for the presence of RHAMM or erk.

Fig.1

Mutation of RHAMM in ES cells

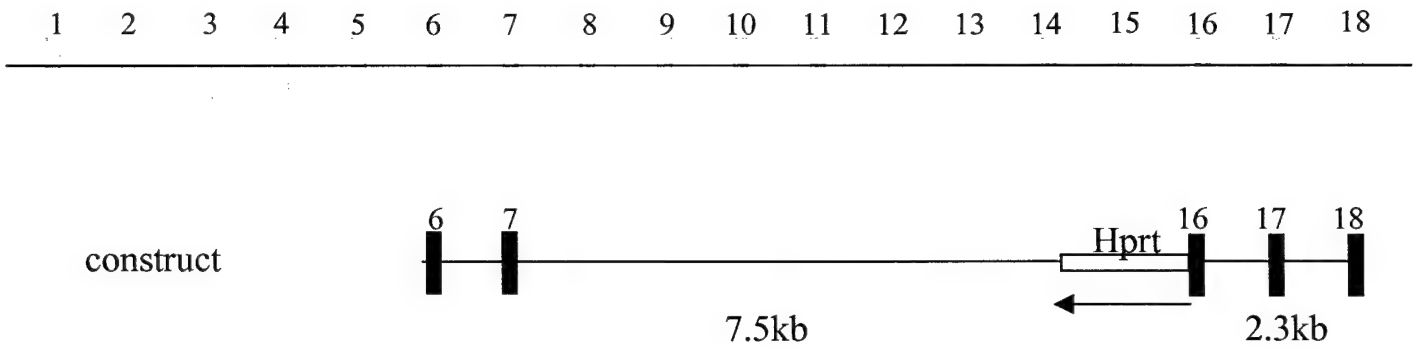
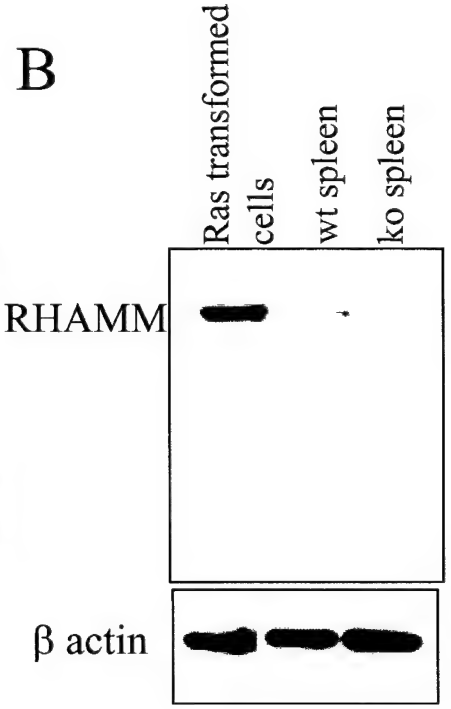


Fig.2 A

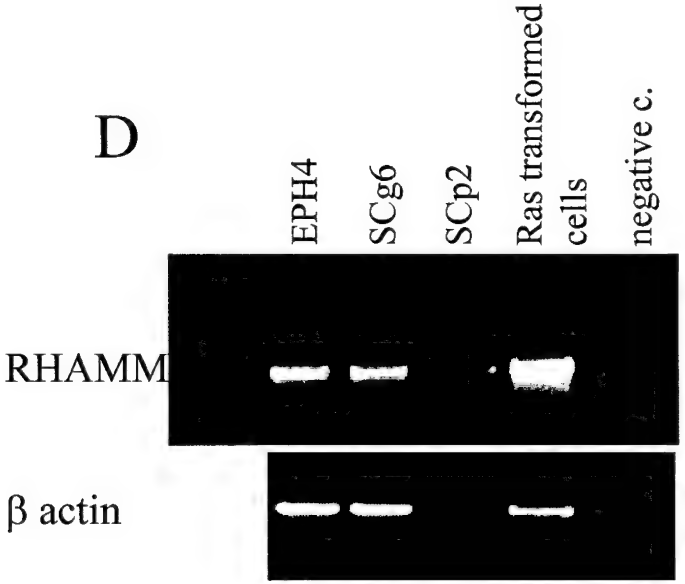

RHAMM -/-
RHAMM+/+
negative c.
RHAMM
β actin

B


Ras transformed cells
wt spleen
ko spleen
RHAMM
β actin

C

D


EPH4
SCg6
SCp2
Ras transformed cells
negative c.
RHAMM
β actin

E

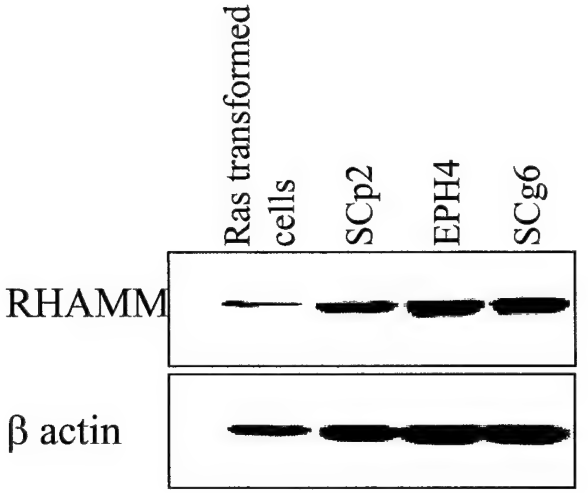
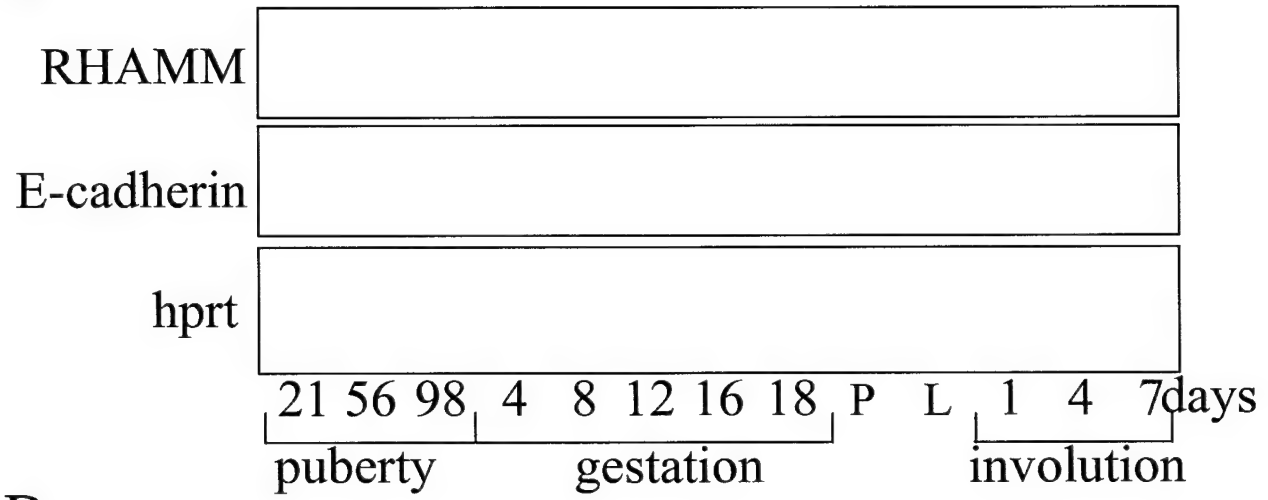

Ras transformed cells
SCp2
EPH4
SCg6
RHAMM
β actin

Fig. 3 A


RHAMM
E-cadherin
hprt
21 56 98 4 8 12 16 18 P L 1 4 7 days
puberty
gestation
involution

B

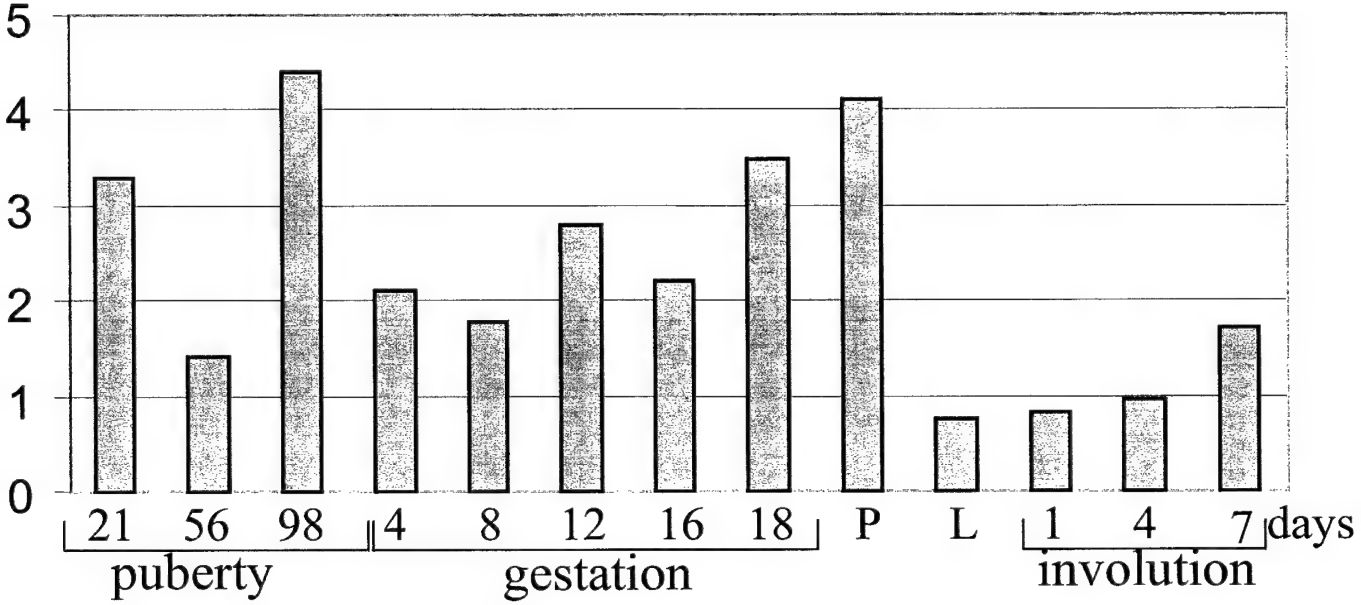

5
4
3
2
1
0
21 56 98 4 8 12 16 18 P L 1 4 7 days
puberty
gestation
involution

Fig.4

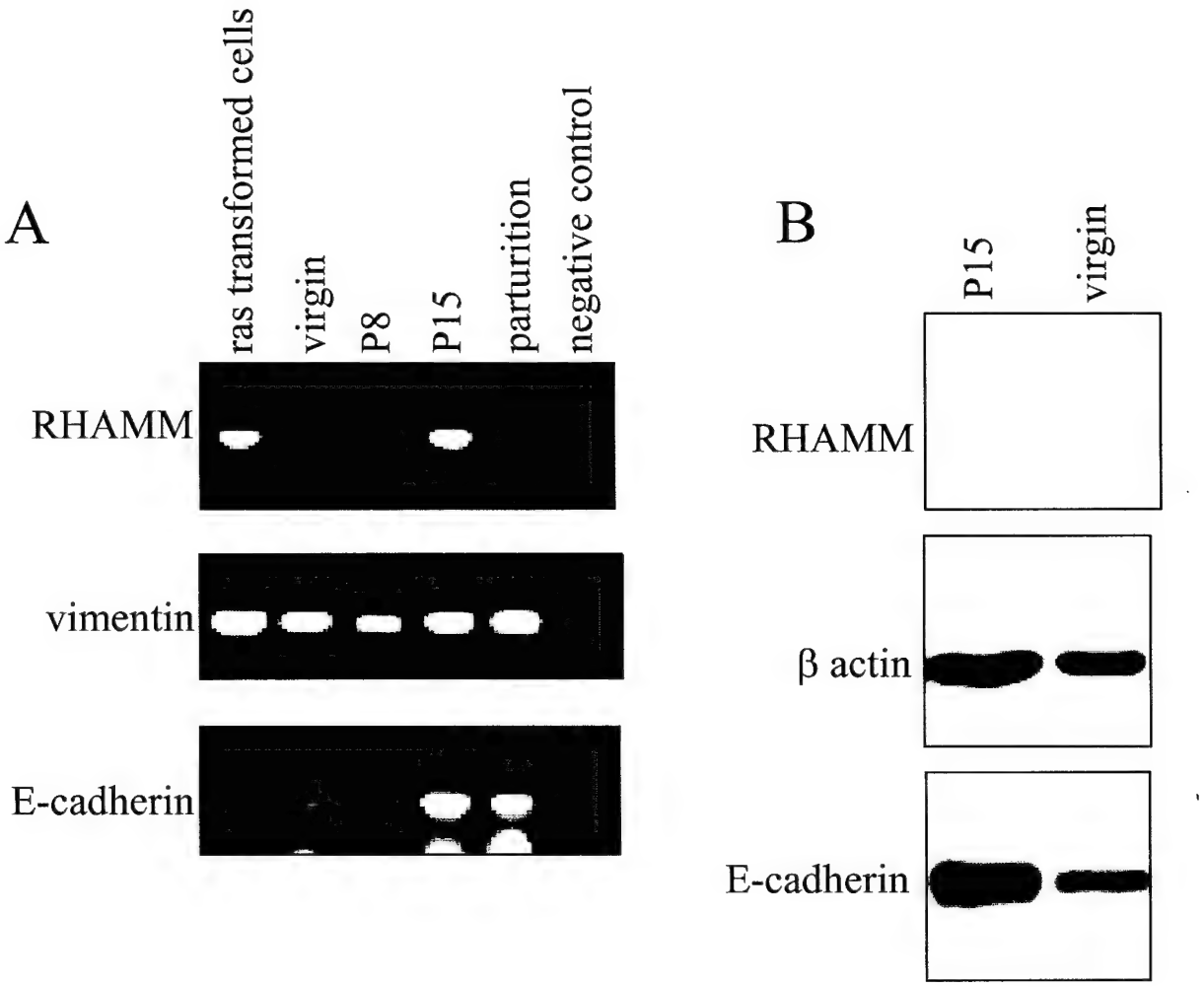
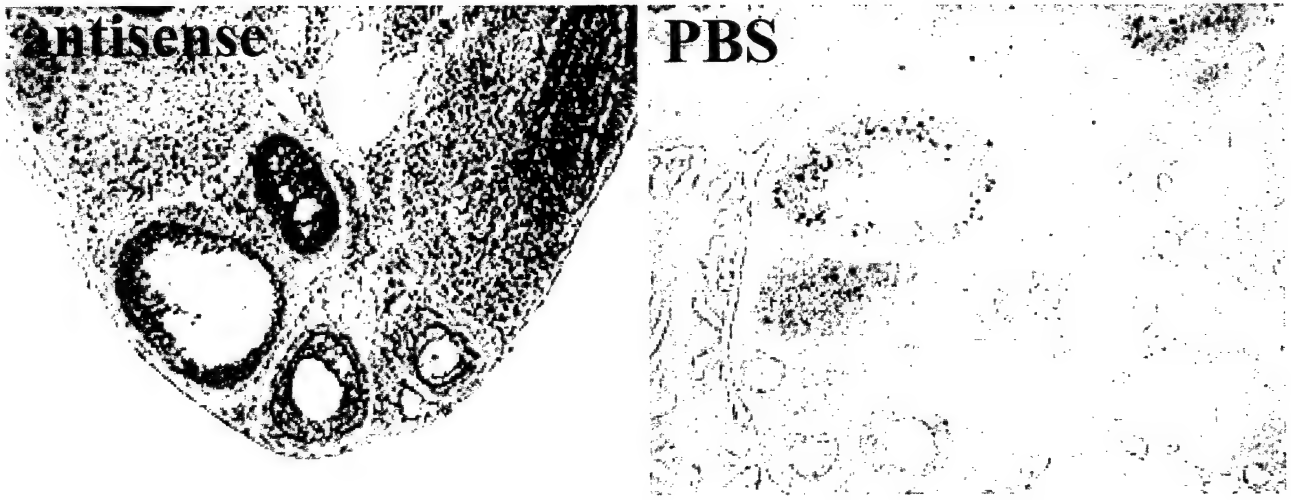

A
ras transformed cells
virgin
P8
P15
parturition
negative control
RHAMM
vimentin
E-cadherin
B
P15
virgin
RHAMM
β actin
E-cadherin

Fig.5

A

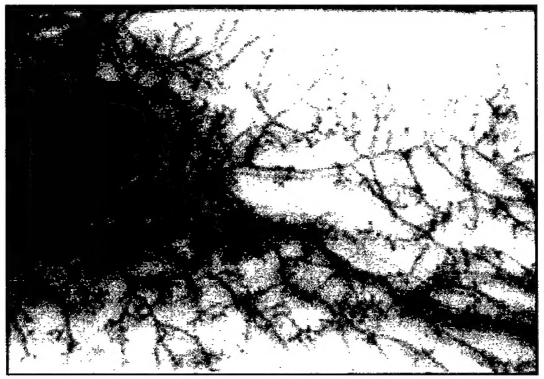


B

antisense sense

Fig.6

wt



ko

Fig.7

wt day 3

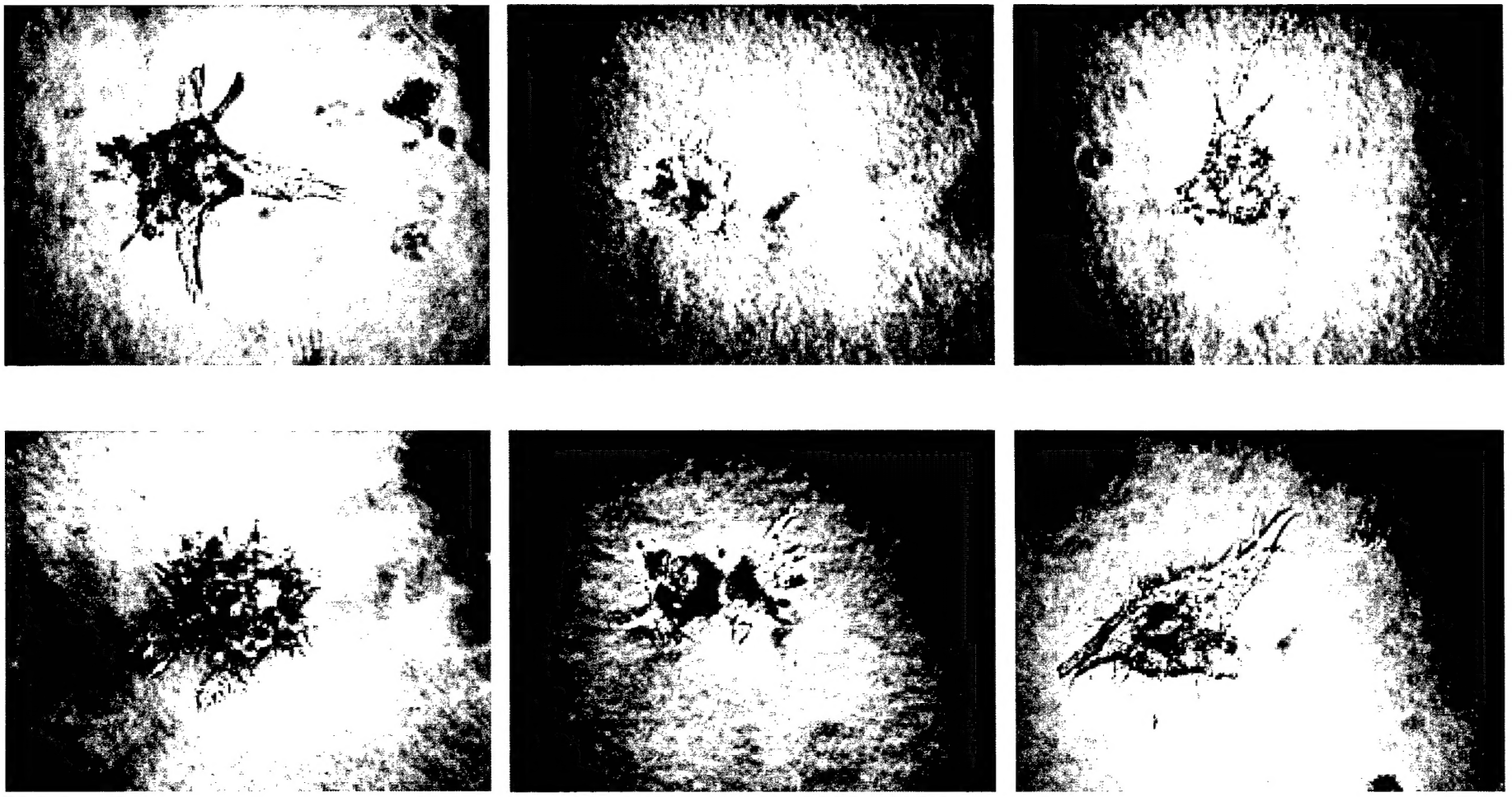


Fig.7

ko day3

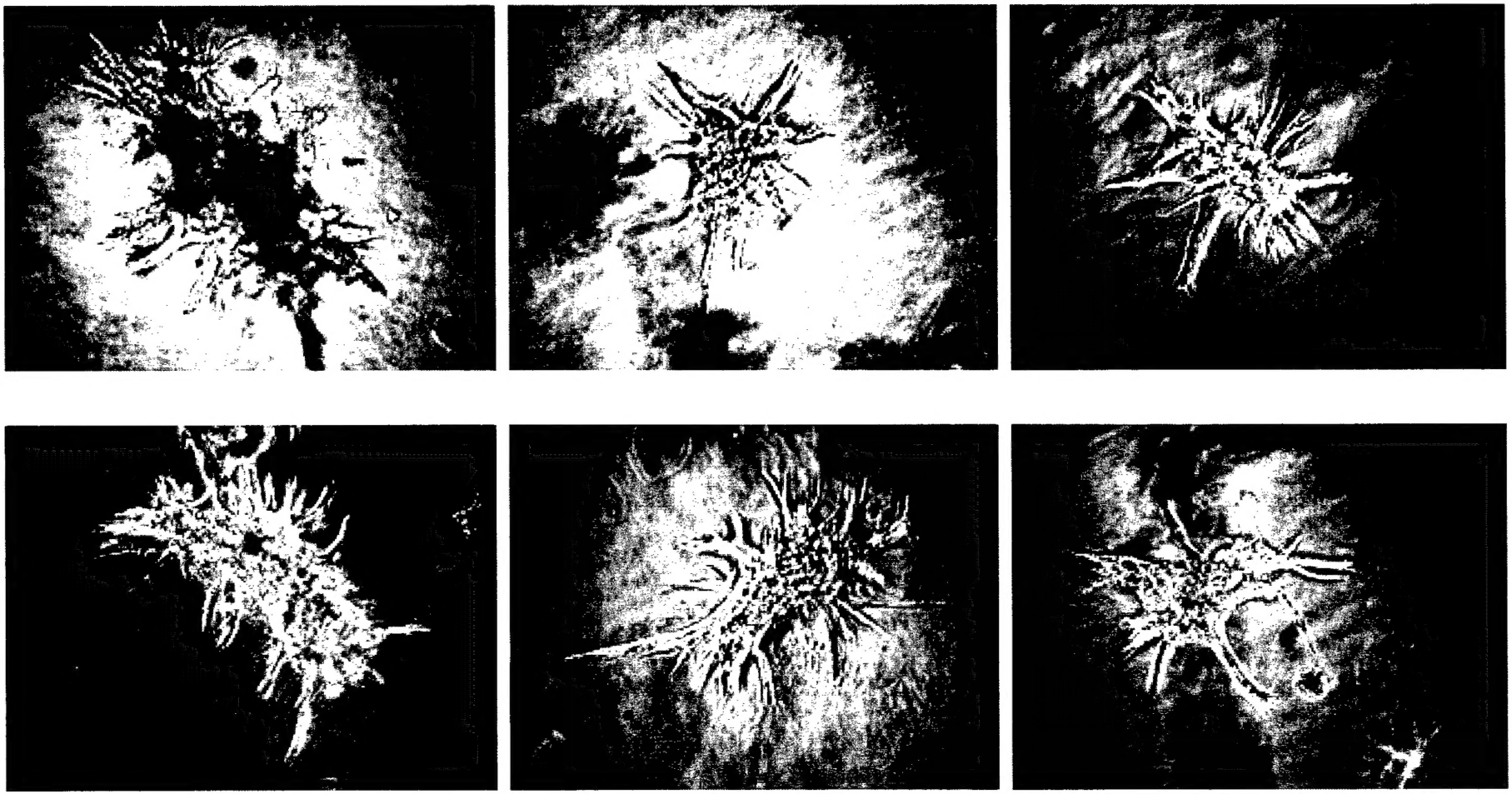


Fig. 8

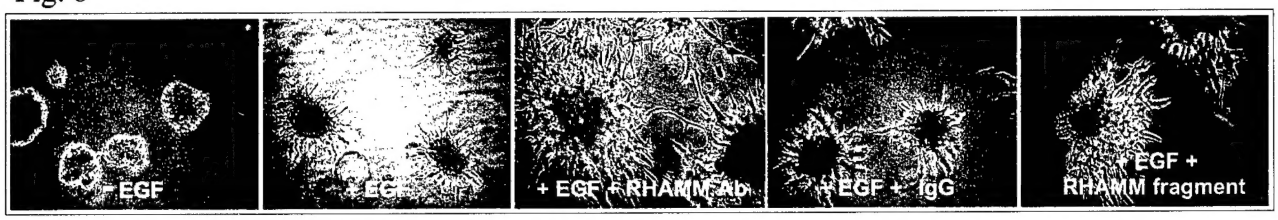
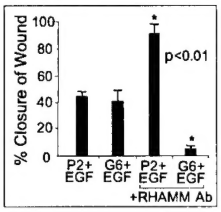


Fig. 9



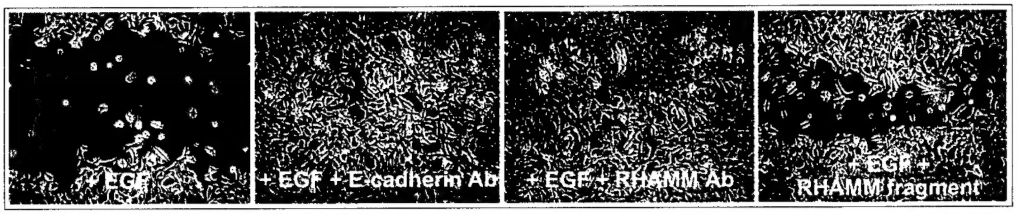


Fig. 10